The present application also relates to a liposome-linked immunosorbant assay which uses liposomes for the detection and quantitation of interacting species with receptors which can be noncovalently immobilized in a format which can be performed manually. The invention also relates to a liposome aggregation immunoassay wherein aggregation of liposomes in solution is detected by an increase in the intensity of light scattered by the solution which is used to determine the concentration of immunoreactive chemicals present in the solution. In this case, liposomes would not have to contain marker molecules, and the assay would be performed in the homogeneous phase by an automated or non-automated method. In another embodiment of the present invention, a manual immunoassay is provided where immobilized antibody is exposed to liposomes containing a visible dye.

In the following immunoassays of the present invention, liposomes were prepared by an injection method from a lipid mixture of dimyristoylphosphatidylcholine (Avanti Polar lipids, Birmingham, Ala.), cholesterol and dicetylphosphate (Sigma Chemicals, St. Louis, Mo.) at a molar ratio of 5:4:1. Varying amounts of N-biotinoyldipalmitoyl-L-phosphatidylethanolamine (B-PE) (Molecular Probes, Junction City, Oreg.) were added to this mixture to achieve concentrations of 0.01 to 1 mol % of total lipid. To prepare liposomes, 2 mol stock lipid mixture in chloroform were evaporated under a stream of nitrogen, and then placed in a vacuum desiccator overnight. The lipid was resolubilized in 0.05 ml of dry isopropanol, and injected with a syringe into 1 ml buffer which was being mixed by vortex. For assays using fluorescent liposomes, this buffer contained the fluorophore, carboxyfluorescein. For assays using a visible dye, the buffer contains dye. Liposomes of uniform size are formed spontaneously by this method. Different methods of preparation can be used, and variation in lipid components is possible. In this case, liposomes are prepared with B-PE so that they can be derivatized noncovalently with biotinylated antibodies or ligands, using avidin as a crosslink. A schematic of this method of derivatization of liposomes is shown in FIG. 5.

To derivatize liposomes, avidin was added to a small volume of liposomes at a concentration to provide a B-PE:avidin molar ratio of 5, and after 2 min, biotinylantitheophylline was added at a molar ratio of antibody:avidin 3. A schematic of this method is shown in FIG. 5.

For the liposome immunosorbant assay (LISA) and the analogous ELISA, liposomes were prepared as above with the B-PE being added to the initial mixture at a concentration of 0.1 mol % of total lipid. Bovine serum albumin (BSA) was covalently coupled to aminopropyl theophylline analog supplied by IGEN, Inc. (Rockville, Md.) with bis(sulfosuccinimidylsuberate) (Pierce Chemical Company, Rockford, Ill.). Monoclonal antitheophylline antibody purchased from American Qualex (La Mirada, Calif.) was purified from ascites fluid with Protein A. Purified antitheophylline was biotinylated with N-hydroxysuccinimidobiotin (NHS-biotin from Pierce) in the following procedure. A 100-fold molar excess of NHS-biotin in DMSO was added to antibody in phosphate-buffered saline (PBS), pH 7.2, and stirred at room temperature for 2 hr., followed by dialysis against PBS. Antitheophylline was noncovalently conjugated to liposomes containing carboxyfluorescein.

The ELISA and LISA assays were based on binding of antitheophylline antibody or antitheophylline-liposomes to theophylline-BSA conjugate which was nonspecifically adsorbed to a plastic support. ELISA was performed in 96-well microtiter plates, and for LISA, disposable polystyrene cuvettes were used. Varying concentrations of theophylline were added to wells and cuvettes to compete for binding to antitheophylline or antitheophylline liposomes. The amount of signal for both the ELISA and the LISA was inversely related to the amount of theophylline added.

Cuvettes and microtiter plates were incubated with theophylline-BSA for 1 hour, washed three times, and then incubated for 1 hr with a buffer solution containing 1% BSA to block any sites on the polystyrene which were not covered with protein. In the LISA, after washing out the blocking solution, solutions of antitheophylline liposomes and theophylline were added to the cuvettes. After 30 min. incubation, unbound antitheophylline liposomes were washed out of the cuvettes. Bound liposomes were solubilized with a detergent solution, and their associated fluorescence was measured at 520 nm by exciting samples at 490 nm. In the ELISA, the wash step which followed blocking was then followed by the addition of antitheophylline plus theophylline. After 60 min., unbound antitheophylline was washed out. This was followed by the addition of peroxidase-labeled secondary antibody (peroxidase antimouse antibody), which served as an indicator of the presence of antitheophylline bound to immobilized theophylline. After an additional 60 min. incubation, the peroxidase-antibody which did not bind to the antitheophylline was washed out. This step was then followed by an additional step: incubation with peroxidase substrate, 2,2'-Azinobis(3-ethylbenzthiazolinesulfonic acid). After 30 min additional incubation, the reaction was stopped by addition of sodium azide to each well. The optical density of each well at 414 nm was measured in an ELISA plate reader. The two assays are compared schematically in FIG. 6.

Almost all aspects of the two assays, such as incubation times at common steps, and concentrations of reagents, were identical. The concentration of antitheophylline used for ELISA was twice the molar concentration of liposomes used in LISA. The ELISA was performed in microtiter plates in a volume of 0.1 ml and the LISA in polystyrene cuvettes with a reaction volume of 0.5 ml and a final volume of 2 ml. From the BSA blocking step to completion, the LISA took approximately 40 min, and the ELISA took about 3.5 hr.

The results of the comparative assays are shown in FIG. 7. The two assays provided qualitatively similar results, but the LISA assay was more sensitive due to increased precision and a greater change in signal with change in concentration. Statistical analysis showed that LISA is at least two orders of magnitude more sensitive than the corresponding ELISA.

In a different embodiment of the present invention, the intensity of scattered light was quantitated as a measure of liposome aggregation in response to a concentration-dependent immunospecific reaction. Liposome aggregation experiments were performed in a spectrofluorimeter using a 450 watt xenon light source by monitoring the intensity of 500 nm light scattered 90° to incident. Liposomes were allowed to incubate for 3 min with avidin before addition of B-Ab. For all experiments, liposomes were present at a concentration of 0.5 nmol total lipid in 2.5 ml, and were continuously stirred.